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Original Article

Evaluation of *ATG-5* and *ATG-7* Gene Expression in the Autophagy Pathway by a Synthetic Circular MicroRNA Sponge Targeting miR-17 and miR-181 in the NALM-6 Cell Line

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ABSTRACT

Introduction: MicroRNAs (miRNAs) can affect cellular processes by modulating the expression of different genes. Blocking specific miRNAs across all cancer types can reduce or prevent cancer cell activity. The present study aimed to investigate the inhibitory effects of MicroRNA sponges (miRSponge) on miR-17-5p and miR-181 in NALM-6 cells, a cellular model of acute lymphoblastic leukemia, and to evaluate their effects on the expression of the autophagy-related genes *ATG-5* and *ATG-7*.

Materials and methods: The present study was conducted from 2021 to 2022 at Mashhad University of Medical Sciences, Iran, in collaboration with the Shiraz Paramedical Faculty, Iran, and focused on a miRSponge designed to target miR-17-5p and miR-181. The present study included four NALM-6 cell groups, including untreated cells as a control, miR-17-5p- and miR-181-sponge-transfected cells, and a vincristine-treated group. The NALM-6 cells were cultured and transfected with a miRSponge targeting miR-17-5p and miR-181. The expression levels of miR-17-5p, miR-181, *ATG-5*, and *ATG-7* were quantified by real-time quantitative PCR, while apoptosis in cells from different study groups was assessed by flow cytometry using Annexin V/7-AAD staining. Cell viability following vincristine treatment was evaluated using MTT assay, and bioinformatics analyses were performed to investigate miRNA-target interactions.

Results: The current results indicated that the developed sponge significantly reduced the levels of these two oncogenic miRNAs in cells by binding to miR-17-5p and miR-181. Furthermore, the activity of *ATG-5* and *ATG-7*, regulated by these two miRNAs, increased following the reduction in the expression of these miRNAs. Late-stage apoptosis was higher in miRSponge-treated cells than in vincristine-treated and control groups, although the difference was not statistically significant.

Conclusion: The present findings indicated that using a miRSponge significantly upregulated the *ATG-5* and *ATG-7* genes and enhanced autophagy and apoptosis in cancer cells. MicroRNA sponges reduced the effects of oncogenic miRNAs in acute lymphocytic leukemia cells.

1. Introduction

Acute leukemia refers to hematological malignancies characterized by an increase in myeloid or lymphoid blasts that progress rapidly due to the undifferentiated nature of leukemic cells[1]. Acute leukemia is the most common cancer in children, with acute lymphoblastic leukemia making up 75-80% of cases[1]. Acute lymphoblastic leukemia (ALL) is the most common cancer in children under 15, accounting for 23% of all cancers and 76% of leukemia cases in

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humans[1]. The ALL is more common among boys, and T-cell acute lymphoblastic leukemia (T-ALL) is about four times more common in boys than in girls[1]. Girls are 1.5 times more likely to develop ALL in their first year[1]. The highest incidence of ALL occurs in developed countries in children ages 2 to 5, and it is more prevalent in individuals of European descent than African descent[1]. Initial signs of adult ALL are related to bone marrow failure[2], leading to pallor, increased heart rate, weakness, and fatigue from anemia[1,2]. Furthermore, murine models of ALL have demonstrated disease-associated manifestations, including splenomegaly, weight loss, failure to thrive, and signs of morbidity during disease progression[3]. Patients may also experience petechiae, bleeding disorders due to thrombocytopenia, and infections due to neutropenia[1,2].

MicroRNAs (miRNAs) are small, single-stranded RNAs approximately 22 nucleotides long[4,5]. MicroRNAs are vital biological regulators that control gene expression at the post-transcriptional stage[4,5]. Over half of the miRNAs in the genome are associated with cancer or genomic instability[6]. MicroRNAs control several cellular processes, including proliferation, differentiation, survival, and apoptosis[7]. miR-17-5p and miR-181 are regulatory miRNAs involved in cell proliferation, survival, and cancer-related pathways, including autophagy[8]. A previous study demonstrated that genetic and epigenetic factors disrupt miRNA levels in cells, impacting cancer development and spread[8]. Based on these findings, analyzing miRNA expression patterns across different cancers can be a valuable diagnostic tool for understanding cancer development and spread[9].

Studies in animal models have demonstrated the diagnostic potential of circulating miRNAs, as tumor-derived miRNAs detected in plasma distinguished cancer-bearing mice from healthy controls, supporting their potential as non-invasive biomarkers for cancer detection[10]. Experimental studies in animal models have further demonstrated the functional importance of miRNAs in leukemia[3]. In a murine model of Notch1-induced T-ALL, miR-19 promoted leukemogenesis and accelerated disease progression, supporting its oncogenic role in lymphoblastic leukemia[11]. Moreover, inhibition of the miR-17/92 cluster using a combined MicroRNA sponge (miRSponge) reduced leukemic cell burden, spleen enlargement, and disease progression in mice with BCR-FGFR1-driven B-cell lymphoblastic leukemia, and prolonged survival[3]. These findings suggested that targeting oncogenic miRNAs may represent a potential strategy for controlling leukemia progression *in vivo*[3].

MicroRNA sponges are synthetic miRNA inhibitors that bind multiple miRNAs, preventing them from reaching their targets[12]. MicroRNA sponges create a bubble between the binding sites, placing miR in a duplex that Argonaute 2 (Ago2) can break down[12]. Additionally, by incorporating multiple binding sites for a miRNA's seed region, a sponge can inhibit an entire miR family, simultaneously inhibiting several miRNAs[12].

Eukaryotic cells use the ubiquitin-proteasome and lysosomal systems to eliminate old organelles and misfolded proteins[13]. Autophagy, including micro-autophagy, chaperone-mediated autophagy, and macro-autophagy, is vital for different functions such as hematopoiesis[14,15]. Several studies indicated that autophagy is crucial for the survival of mouse hematopoietic stem cells[16,17]. Removing proteins such as ATG-5, ATG-7, and FIP200 impairs their survival and transplantation[16,17]. However, excessive or dysregulated autophagy can also lead to programmed cell death[18,19]. Additionally, autophagy can have dual roles in tumorigenesis, acting as an inhibitor and an inducer depending on the cancer stage[20,21].

Despite the established role of autophagy in hematological malignancies, the regulatory effects of miR-17-5p and miR-181 on key autophagy-related genes remain poorly understood in NALM-6 cells. The NALM-6 is a human B-cell precursor acute lymphoblastic leukemia (B-ALL) cell line[22] and was selected as a model to investigate the effects of targeting these miRNAs on the expression of autophagy-related genes. In particular, the potential of a synthetic circular miRSponge targeting these miRNAs to modulate *ATG-5* and *ATG-7* expression has not been adequately investigated. Therefore, the present study aimed to evaluate the inhibitory effects of miR-17-5p and miR-181 on the expression of the *ATG-5* and *ATG-7* genes in NALM-6 cells.

2. Materials and Methods

2.1. Ethical approval

The study protocol was validated by the Ethics Committee of the Mashhad University of Medical Sciences (MUMS) and the Institutional Review Board of Mashhad University Medical Center, Iran (Ethics Code: IR.MUMS.MEDICAL.REC.1399.481).

2.2. Experimental design and cell grouping

This study was conducted at the Mashhad University of Medical Sciences, Iran, in collaboration with the Science and Research Center of the Shiraz Paramedical Faculty, Iran, during 2021-2022. The experimental trial comprised four NALM-6 cell groups, including untreated cells as controls, cells electroporated with sponge constructs targeting either miR-17-5p or miR-181, and a vincristine-treated group. Following 24 hours of incubation, apoptosis was quantified by flow cytometry (five replicates per condition), and real-time PCR was used to determine transcript levels of the autophagy-related genes (*ATG-5* and *ATG-7*). This design enabled direct comparison of the effects of OncomiR suppression with those of standard chemotherapy, specifically regarding the induction of programmed cell death and the transcriptional regulation of autophagic mediators. After 48 hours, cells were stained with an Annexin V kit for flow-cytometric detection of apoptosis.

2.3. Cell culture

The NALM-6 cell line (DSMZ no. ACC128; Pasteur Institute; Iran) was selected as an acute ALL cell line to study the impact of miR-17-5p and miR-181 on preventing cancer cell death by targeting *ATG-5* and *ATG-7*. Cell culture, freezing, and thawing procedures were carried out in accordance with Sigma-Aldrich guidelines, with protocols

adapted from the study by Wang et al.[23]. The NALM-6 cell line was chosen as a well-characterized B-ALL model with wild-type p53 and a stable karyotype, providing a clean genetic background for mechanistic studies[24]. The rapid growth and high transfection efficiency of NALM-6 facilitate miRNA manipulation, while its partial sensitivity to vincristine enables clinically relevant comparisons. These characteristics collectively made NALM-6 an optimal choice for investigating the regulation of apoptosis and autophagy.

2.4. Collecting cells for RNA extraction

When the culture reached the proper confluency, cells were washed with PBS buffer, and 2.5-3 million cells were placed in 250 µL of PBS buffer in each tube for RNA extraction and stored at -70°C[25].

2.5. Purification of total RNA

The manual Trizol/chloroform extraction method was chosen to isolate total RNA, based on the methodology of Chomczynski and Sacchi[26]. This method consisted of five steps, starting with RNA stabilization and phase separation using Trizol (Invitrogen, USA). Then, precipitate RNA with cold isopropanol (Merck, Germany), wash the RNA with cold 75% ethanol, dissolve the RNA in RNase-free water (Cinnagene, Iran), and finally assess RNA concentration, purity, and quality with a NanoDrop device (Hellma, Denmark) and compute the OD ratios at 260/280 nm and 260/230 nm. The purified total RNA with acceptable purity and quality was stored at -70°C.

2.6. Production of specific cDNA

Specific primers (Exiqon, USA) were used to generate cDNA for miR-17-5p and miR-181 in the present study. The cDNA was synthesized using an Exiqon (USA) kit, following the provided protocol. Additionally, cDNA for *ATG-5* and *ATG-7* mRNA and 5S ribosomal RNA (5S-rRNA) was produced using the same kit (Exiqon, USA) protocol. The primers utilized for cDNA synthesis are detailed in Table 1.

Table 1. MicroRNA-specific primers for cDNA synthesis reaction

miRNAs	Primer Sequence*
miR-181	5´ AACAUUCAUUGCUGUCGGUGGGU 3´
miR-17-5p	5´ CAAAGUGCUUACAGUGCAGGUAG 3´

* The primer sequences reported in this study were independently designed and selected by the authors based on the bioinformatic analyses performed for the present study.

2.7. Real-time PCR

Metabion (Germany) created *ATG-5*, *ATG-7*, GAPDH, miR-17-5p, miR-181, and 5s-rRNA primers. These primers were tested using an Exiqon kit (USA) and real-time PCR. Real-time PCR was performed using an Applied Biosystems StepOne™ Real-Time PCR System (Thermo Fisher Scientific, USA) with the accompanying StepOne Software v2.1. The thermal cycling protocol comprised an initial denaturation step at 95 °C for five minutes, followed by 42 cycles of denaturation at 95 °C for five seconds, annealing at 62 °C for 20 five seconds, and extension at 72 °C for 30 seconds. The tools and software were used according to the manufacturer's instructions (Thermo Fisher Scientific, StepOne™ Real-Time PCR System Getting Started Guide) and the guidelines adapted from the study of Mortazavi et al.[27]. The primer sequences designed and used for real-time PCR were listed in Table 2.

Table 2. Primer sequences used for real-time PCR for gene expression investigation

Target	Primers sequences*
ATG-5	F: 5'-GGCCATCAATCGGAAACTC-3' R: 5'-AGGTCTTTCAGTCGTTGTCT-3'
ATG-7	F: 5'-ATTGCTGCATCAAGAAACCC-3' R: 5'-GATGGAGAGCTCCTCAGCA-3'
GAPDH	F: 5'-GGACTCATGACCACAGTCCA-3' R: 5'-CCAGTAGAGGCAGGGATGAT-3'
miR-17-5p	F: 5'-TCTAGATCCCGAGGACTG-3' R: 5'-ATCGTGACCTGAACC-3'
miR-181	F: 5'-GCGGCAACATTCAACGCTGTCGGTGAGT-3' R: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTG-3'
5s-rRNA	F: 5'-GGCCATACCACCCTGAACGC-3' R: 5'-CAGCACCCGGTATTCCCAGG-3'

F: forward primer, R: reverse primer. * The primer sequences reported in this study were independently designed and selected by the authors based on the bioinformatic analyses performed for the present study.

2.8. MicroRNA sponge

The miRSponge was an inhibitor designed by Biomatic Company (Canada) to target two specific miRNAs. The miRSponge was cloned into the eukaryotic vector Psi-check2 between two restriction sites related to the XhoI (5') and NotI (3') enzymes. To design this miRSponge, four miRNA binding sites (MBS) were considered for binding miR-181 and four binding sites for miR-17-5p, which were located on the miRSponge consecutively. The sequence design and production of the miRSponge were carried out by Biomatic company (Canada). The miRSponge was designed and *in silico* tested using the miRNAsong bioinformatics tool to generate a sponge specific to the target miRNAs and to assess potential off-target interactions[28]. The complete sequence of the miRSponge and the sequences related to the binding site of miR-181 and miR-17-5p are presented in Table 3.

Table 3. Sequences related to the miRSponge and the targets

Presentation	Sequence
Specific miRSponge sequence	CTCGAGACCCACCGACATTCTGAATGTTAATTACCCACCGACATTCTGAATGTTAATTACCCAC CGACATTCTGAATGTTAATTACCCACCGACATTCTGAATGTTAATTCTACCTGCACTCCCGCAC TTTGAATTCTACCTGCACTCCCGCACTTTGAATTCTACCTGCACTCCCGCACTTTGAATTCTAC CTGCACTCCCGCACTTTGAATTAATTGCGGCCGC
miR-181 binding site sequence on the miRSponge	ACCCACCGACATTCTGAATGTT
miR-17-5p binding site sequence on the miRSponge	CTACCTGCACTCCCGCACTTTG

2.9. Plasmid transfer to bacteria

The *Escherichia coli* (*E. coli*) DH5α strain (Pasteur Institute, Iran) was used to transform the Psi-check2 vector (Promega, USA) containing miRSponge into bacteria[29]. This transformation method was used to propagate and obtain sufficient quantities of the desired recombinant plasmid. Therefore, a cold method was employed, using cold calcium chloride (CaCl2; Kimya Teb, Iran), as previously described by Chan et al.[30].

2.10. Plasmid extraction

Plasmid extraction was performed from the transformed *E. coli* DH5α cells using the Yekta Tajhiz Azma (Iran) plasmid extraction kit according to the manufacturer's instructions. The extracted plasmid was then checked for concentration using a NanoDrop device. Additionally, the quality and purity of the plasmid were examined using the OD ratio at wavelengths of 260/280 nm.

2.11. Plasmid transfer to NALM-6 cells

An electroporation device (Bio-Rad, USA) was used to transfer the Psi-check2 vector into NALM-6 cells. After experimenting with different conditions and modified protocols adapted from the study of Brouns et al.[22], the optimal program was found to use a voltage of 269V, a capacitance of 1050 µF, a plasmid concentration of 22 µg/µl, and 4 million cells. Briefly, NALM-6 cells (4×10^6) were mixed with 22 µg/µL of Psi-check2 plasmid DNA and subjected to electroporation using a Bio-Rad electroporator at 269 V and 1050 µF. Following electroporation, the cells were immediately transferred to complete culture medium and incubated under standard cell culture conditions, as previously described for electroporation of NALM-6 cells[22].

2.12. Apoptosis assay

The apoptosis assay was conducted using the BD Annexin V/7-AAD kit (BD, USA) and following its protocol to assess the extent of apoptosis induced in NALM-6 cells transfected with the inhibitor. The apoptosis assay used Annexin V/7-AAD staining to measure apoptosis. The test was conducted after 48 hours of transfection in two groups of NALM-6 cells. One group of NALM-6 cells was transfected with psi-Check2 vectors containing the inhibitory fragment as a test group. In contrast, the control group did not receive the plasmid and underwent the same electroporation electrical pulse conditions (269 V and 1050 µF).

2.13. MTT assay

Vincristine sulfate (Sigma-Aldrich, USA) was used as a positive control due to its established anticancer activity and its ability to induce cell death by interfering with microtubule polymerization. The effect of different vincristine concentrations on NALM-6 cell viability and proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) assay after 24, 48, and 72 hours of treatment. A total of 20×10^3 cells were seeded per well in RPMI-1640 culture medium (Biowest, USA).

2.14. Bioinformatics tools

Different bioinformatics tools and databases were used in the present study, including TargetScanHuman v8.0, UCSC Genome Browser v482 (GRCh38/hg38), miRanda v3.3a, and miRTarBase Release 9.0. TargetScanHuman and miRanda to predict potential miRNA target genes, the UCSC Genome Browser for genomic and transcript annotation, and miRTarBase to identify experimentally validated miRNA–target interactions. miRNAsong (2016 release) was used to design and *in silico* evaluate the miRSponge construct, and STarMir (2014 release) was used to assess miRNA binding sites and associated thermodynamic features[31].

2.15. Statistical analysis

The present data were analyzed using GraphPad v7.05 software. The t-test was used to compare the mean values of the study groups. The significance level in the study was set at a p-value less than 0.05 ($p < 0.05$).

3. Results

The present findings were divided into three sections; quantifying the expression levels of specific miRNAs, assessing alterations in the expression of the target genes corresponding to the studied miRNAs, and analyzing the extent of cell death and apoptosis in the NALM-6 cell line upon transfection with the miRSponge, compared with the control group.

3.1. MicroRNA expression levels using real-time PCR

Figures 1A and 1B clearly illustrated a significant decrease in the expression of two miRNAs, miR-181 and miR-17-5p ($p < 0.05$), which are known as cellular solid OncomiRs in transfected cells compared to the non-transfected group. The current results indicated that vincristine did not affect the expression of miR-181 ($p > 0.05$; Figure 1C), while vincristine significantly decreased the oncomiR miR-17-5p expression compared to the control group ($p < 0.05$), which did not receive vincristine ($p < 0.05$; Figure 1D).

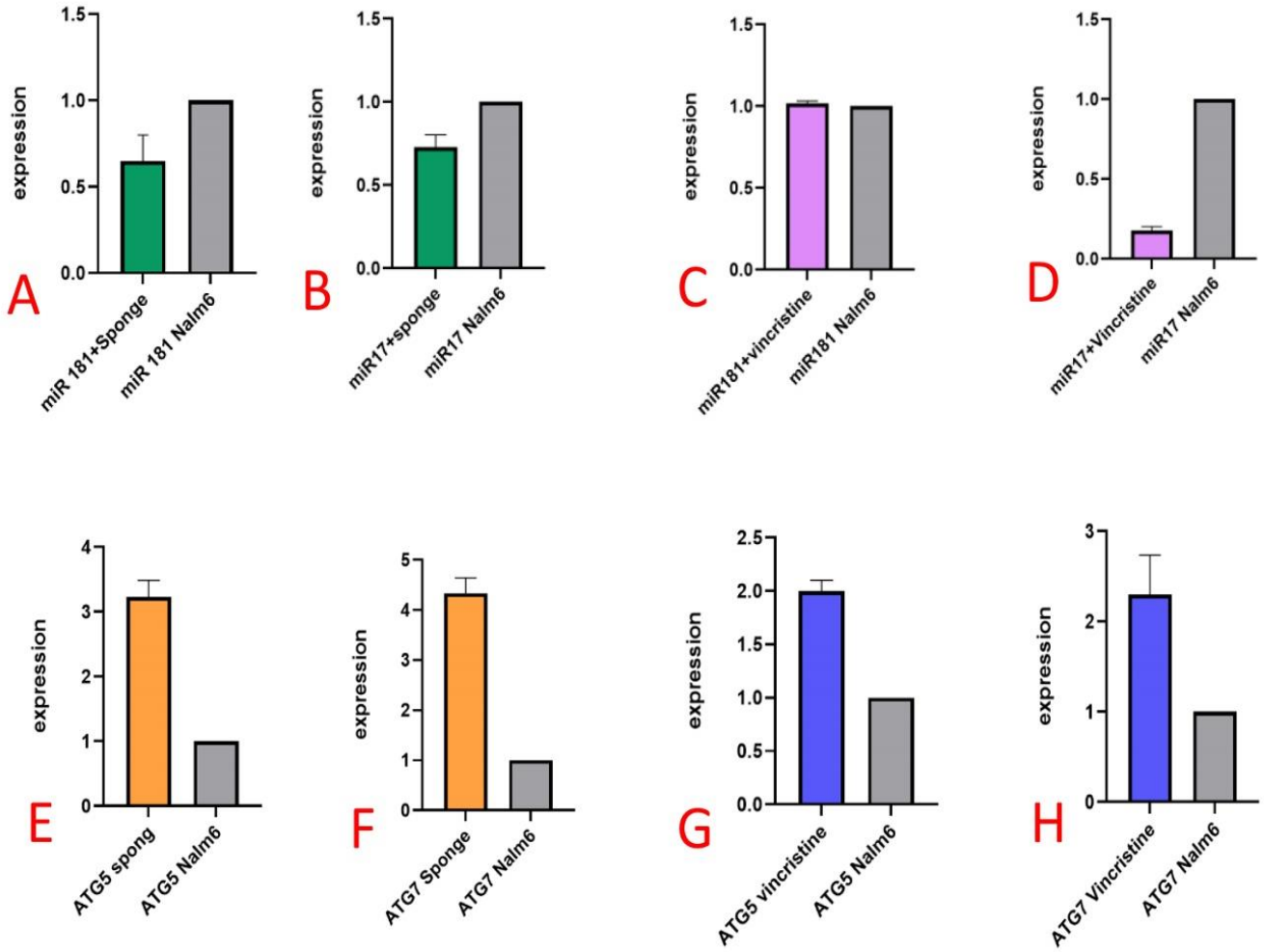

Figure 1. Levels of miR-181, miR-17-5p, *ATG-5*, and *ATG-7* expression using real-time PCR. **A:** Sponge transfection miR-181 expression in NALM-6 cells (green bar) compared to the non-transfected control, **B:** Sponge transfection miR-17 expression in NALM-6 cells (green bar) compared to non-transfected cells (control), **C:** miR-181 expression in NALM-6 cells treated with vincristine (purple bar) compared to control cells, **D:** miR-17 expression in NALM-6 cells exposed to vincristine (purple bar) compared to control cells, **E:** *ATG-5* gene expression in sponge-transfected cells (orange) compared to non-transfected NALM-6 cells, **F:** *ATG-7* gene expression in sponge-transfected cells (orange) compared to non-transfected NALM-6 cells, **G:** *ATG-5* gene expression in NALM-6 cells exposed to vincristine (blue bar) compared to control cells, **H:** *ATG-7* gene expression in NALM-6 cells exposed to vincristine (blue bar) compared to control cells. Gray bar: Control cells.

3.2. Gene expression changes using real-time PCR

Changes in gene expression in NALM-6 cells after treatment with a sponge and vincristine were assessed. The present results indicated that *ATG-5* and *ATG-7* expression levels in transfected cells were significantly higher than in non-transfected NALM-6 cells, which served as the control group ($p < 0.05$). *ATG-5* and *ATG-7* demonstrated significant changes compared to non-transfected NALM-6 cells ($p < 0.05$; Figures 1E and 1F). Based on the results of real-time PCR, vincristine significantly increased the expression of the two autophagy genes, *ATG-5* and *ATG-7*, compared to the untreated NALM-6 cells ($p < 0.05$; Figures 1G and 1H).

3.3. Cell death triggered by MicroRNA sponge by flow cytometry

The flow cytometry results demonstrated a higher proportion of apoptotic and dead cells 48 hours after transfection with the miRSponge and vincristine treatment (Figures 2 and 3). The current findings indicated that the late-stage apoptotic cell count was higher in samples treated with the miRSponge than in those treated with vincristine or the control group; however, no statistically significant difference was observed ($p > 0.05$). These findings demonstrated an elevated level of apoptosis induced by the specially designed miRSponge.

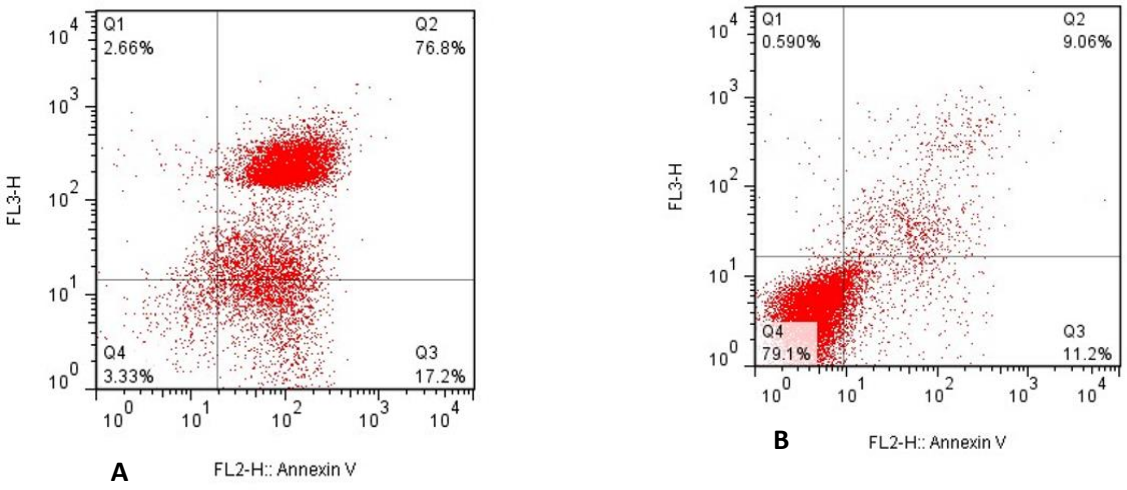

Figure 2. Scatter plots from flow cytometry regarding apoptosis in sponge-transfected cells after 48 hours. **A:** The test group received the vector containing the inhibitor fragment. **B:** The control group received a pulse without the vector. Apoptotic cells were stained with Annexin V and 7-AAD dyes. The lower-left region represents live cells, the lower-right area represents live cells in early apoptosis, the upper-right area represents late-stage apoptosis, and the upper-left area represents necrotic cells.

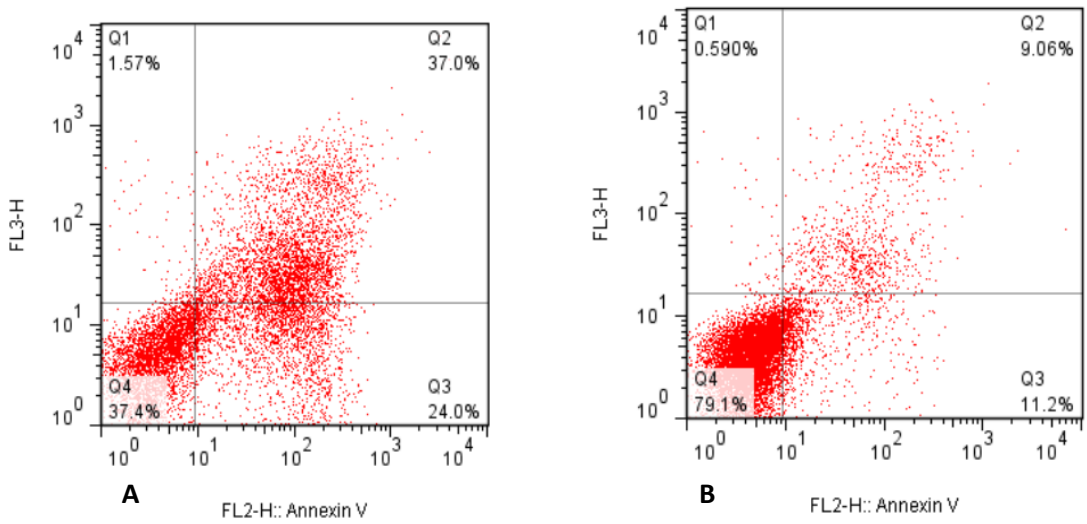

Figure 3. Scatter plots from flow cytometry regarding the vincristine-treated cells undergoing apoptosis after 48 hours. **A:** The flow cytometry results of cells treated with vincristine. **B:** The control group. Apoptotic cells were stained with Annexin V and 7-AAD dyes. The lower-left area represents live cells, the lower-right area represents early apoptosis, the upper-right area represents late apoptosis, and the upper-left area represents dead cells.

4. Discussion

Alterations in miRNA expression and function affect cellular signaling pathways, which are believed to be important contributors to changes in protein structure that lead to cancer and other illnesses[32]. On the other hand, miRSponges are a viable approach to modifying the activity and impact of miRNAs.

Previous studies on miRsponge design have noted that miRsponges containing a mismatch at the MBS center exhibit superior performance compared to miRsponges that form a perfect match with their target miRNA, resulting in more robust inhibitory properties[33]. In the present study, short AATT sequences were placed between the MBSs during miRNA sponge design to minimize nonspecific interactions and provide sufficient space for simultaneous miRNA binding. The design of short AATT sequences between the MBSs was consistent with Jung et al.[34], who have used short AATT spacers between adjacent MBSs to minimize nonspecific interactions. Furthermore, in the present study, to generate a central bulge in the miRsponge duplex, one nucleotide was deleted, and three nucleotides were substituted within the MBS. A similar bulged-MBS design has previously been reported and proposed to improve sponge stability and reduce susceptibility to cleavage by the RNA-induced silencing machinery[33]. Additionally, the intracellular degradation systems assisted in the destruction of miRNA[35]. In the present study, the Psi-check2 vector used for transfection contained an ampicillin-resistance (*AmpR*) gene for bacterial selection and a *Renilla*

luciferase reporter gene in the 3′ UTR, which was driven by the T7 promoter. The *Renilla luciferase* reporter enabled detection and quantification of reporter activity in cells following transfection[36].

Several studies have demonstrated the involvement of miRNAs in cancer. For instance, Ji et al.[37] reported the impact of miR-181 in hepatocellular carcinoma, while Scherr et al.[38] observed higher miR-17 expression in the NALM-6 cell line. Previous studies partially confirmed the selection of miR-17-5p and miR-181 in the present study and revealed their effects on carcinogenesis. The use of bioinformatics tools aided in selecting these miRNAs for further investigation. Furthermore, Piya et al.[39] demonstrated that upregulating genes such as ATG-7 can enhance autophagy and promote apoptosis in human acute myeloid leukemia (AML) cells, including HL-60 cells. Conversely, knockdown of ATG-7 in a disseminated human AML mouse model has been shown to improve survival following chemotherapy[39]. The findings of Piya et al.[39], obtained in AML cell and animal models, were broadly consistent with the present results, which indicated that increased *ATG-7* expression was associated with enhanced autophagy and apoptosis. However, further animal model studies are needed to validate the findings of the present study within the context of the miRSponge-based approach. Melo Maia et al.[40], indicated that miRSponge decreased miR-17 levels in the SW954 cell line, a human vulvar squamous cell carcinoma cell line, a miRNA associated with vulvar cancer. The findings of Melo Maia et al.40 partially align with the present results, providing insight into the efficacy of miRSponge in decreasing targeted miRNA levels in cells. Jung et al.[34] explored the impact of miRSponge on the inhibition of several miRNAs, including miR-21, miR-155, miR-221, and miR-222, in human breast and pancreatic cancer cell lines. The findings of Jung et al.[34] were consistent with the present results and underscored the potential of miRSponge as a robust tool for inhibiting diverse miRNAs and guiding cancer cells toward apoptosis. However, Jung et al.[34] used multiple cell lines, which was in contrast to the present study. Jung et al.[34] observed a reduction in luciferase levels as an indicator of miRNA inhibition by miRSponge, suggesting that miRSponges containing multiple target sites may provide greater efficacy in inhibiting miRNA activity. Despite these disparities, including differences in targeted miRNA types, vector types, cell induction methods, and cell line types, the present study and the report by Jung et al.34 demonstrate that miRSponge can effectively alter miRNA populations within cells. However, Jung et al.[34] found that some of the target proteins expected to show increased expression following miRSponges induction did not show increased expression. This discrepancy could be attributed to the presence and concurrent effects of multiple miRNA types during the expression of these proteins.

In the present study, the real-time PCR analysis confirmed the expected increase in the expression of the target genes, *ATG-5* and *ATG-7*. The current results indicated that cells containing the miRSponges had lower miR-181 expression than vincristine-treated cells, whereas the reduction in miR-17-5p expression was less pronounced. The decreased miR-181 expression may have contributed to increased *ATG-5* and *ATG-7* expression and, consequently, enhanced autophagy-associated apoptosis and cell death in sponge-transfected cells. This observation was consistent with previous findings of Tekirdag et al.[41], who indicated that miR-181A negatively regulated autophagy by directly targeting *ATG-5*, whereas inhibition of miR-181A increased *ATG-5* expression and autophagic activity.

5. Conclusion

Transfecting ALL cancer cells with a miRSponge to inhibit miR-181 and miR-17-5p significantly increased the amount of autophagy and apoptosis in NALM-6 cells. The present study demonstrated that deploying a miRSponge led to a more significant upregulation of *ATG-5* and *ATG-7* associated with miR-181 and miR-17-5p. The current results indicated that a miRSponge can stimulate cancer cells to enhance autophagy and apoptosis. MicroRNA sponges can potentially mitigate the effects of oncogenic microRNAs and support leukemia treatment. The present study had several limitations. The experiments were performed in a single leukemia cell line (NALM-6), limiting the generalizability of the findings. Moreover, the study was conducted *in vitro*, and the therapeutic effects of the miRSponge were not evaluated in animal models. Although changes in *ATG-5* and *ATG-7* expression were observed, their direct roles in autophagy and apoptosis were not confirmed. Further studies are needed to evaluate the effects of miR-17 and miR-181 inhibition on other cellular processes, including migration and hypoxia, and to assess potential non-specific miRNA interactions. Animal studies are also required to further investigate the therapeutic potential of miRSponge in cancer.

Declarations

Acknowledgments

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Authors' contributions

Faezeh Barzegar was responsible for the original draft, review and editing, conceptualization, data curation, formal analysis, and investigation, and contributed equally to the manuscript with Hojjat Ghahvechi. Hojjat Ghahvechi was responsible for the original draft writing, review and editing, conceptualization, data curation, formal analysis, and investigation. Gholamhossein Tamaddon was responsible for corresponding author, review and editing of the manuscript, conceptualization, data curation, formal analysis, investigation, and methodology. Zahra Darvish Khalilabadi was responsible for the original draft, review and editing, formal analysis, and investigation. Kamyar Kiamarzi was responsible for the writing of the original draft, review and editing, Data curation, Formal analysis, and Investigation.

Mohammad Reza Keramati was the corresponding author and was responsible for review and editing of the manuscript, as well as conceptualization, investigation, methodology, project administration, and supervision.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

The authors declared that this manuscript is original and has not been previously published or submitted for publication elsewhere. The authors confirmed that no plagiarism or duplicate submission has occurred and that all data presented in this manuscript are original, accurate, and honestly reported, and the authors take full reposibility of the data originality before submission and during the peer review process. All sources have been appropriately acknowledged and cited. No generative artificial intelligence tools were used in the design, analysis, interpretation, or writing of this manuscript.

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